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Research Article

ANTITRYPANOSOMAL ACTIVITY OF METHANOLIC EXTRACT OF KHAYA SENEGALENSIS TREE BARK AGAINST TRYPANOSOMA EVANSI

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ABSTRACT
In search of new trypanocidal compounds from medicinal plants, *Khaya senegalensis* root bark was extracted with methanol and screened against *Trypanosoma evansi* for *in vitro* trypanocidal activity on Vero cell line maintained in Dubecco's Modified Eagle Medium (DMEM). It was seeded in flat bottom ELISA plates supplemented with fetal calf serum (FCS) 20-40% and incubated at appropriate conditions for more than 12 h. *In vitro* cytotoxic effects of methanolic plant extract (MPE) of *K. senegalensis* was done on same medium, without FCS, as mentioned above at concentrations (1.56-100 µg/ml) for 72 h. The adhered cells were stained with a drop of crystal violet in phosphate buffered solution. *In vitro*, at 250 µg/ml, there was immobilization, reduction of average trypanosomes counts and complete killing of trypanosomes at 6 h of incubation, which was equivalent to diminazine aceturate (DA) 50 µg/ml (standard drug) at 4 h. *In vitro* cytotoxic effects such as distortion, swelling, sloughing and death of cells from the bottom of affected cells were observed. Both MPE and DA were cytotoxic to Vero cells at all concentrations except 3.13-1.56 and 6.25-1.56 µg/ml, respectively

Key Words: *Khaya senegalensis, Trypanosoma evansi, Trypanocidal, In vitro Cytotoxicity*

INTRODUCTION
Trypanosomosis, an important blood protozoan zoonotic disease, is caused by flagellate parasites of the genus *Trypanosoma*. In Africa, the estimated losses as a result of the disease in agricultural production amounted to 3 billion pounds annually (Hursey, 2000). Resistance to current trypanocides is on the increase as reported in endemic regions globally where the disease is prevalent (Freiburghaus *et al*. 1996). Different parts of *Khaya senegalensis* (Meliaceae) have been used in Malian traditional medicine as antiparasitic drug (Ahua *et al*. 2008). Biological activities such as antimicrobial and antifungal by limonoids from *K. senegalensis* have been reported (Abdelgaleil *et al*.2005). Recent ethno pharmacology and ethno medicine revealed that several medicinal plants possess trypanocidal compounds, which may hold the key for a future potential trypanocides (Wurocheke and Nok, 2004; Shaba *et al.* 2006 and Shaba *et al*. 2007). Thus, new approach and trypanocides are highly needed to combat trypanosomes. On this backdrop, *K. senegalensis* root bark extract was screened against *T. evansi* for possible antitrypanosomal activity.

MATERIALS AND METHODS
Chemicals
Silica gel-G for thin layer chromatography (TLC), solvents (hexane, chloroform, methanol, acetic acid, and ethyl acetate) for extraction of plant material and selection of suitable solvent system used in development /analysis of TLC plates, vanillin for spray and iodine for detection of bioactive constituent(s) on applied extract on TLC plates. All the chemicals were of high analytical grade and purchased from E. Merck, India.

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Plant Material

K. senegalensis (Juss**)** root bark was obtained from herbarium, Department of Biological Science, Faculty of Sciences, Ahmadu Bello University, Nigeria.

Extraction

Twenty **grams** of the dried *K. senegalensis* root bark were pounded into powder with laboratory pestle and mortar. Powdered *K. senegalensis* was cold extracted thrice with 200 ml of methanol according to Stahl (1969). The filtrates were dried at 37 C and stored at 4 C until used.

Thin Layer Chromatography (TLC)

Aliquots (0.2ml) of extract were applied on TLC plates, dried under room temperature and immersed inside the solvent systems in glass jar. Solvent systems tried are listed in the next subsection. This was done to detect, if any, the presence of bioactive constituents in applied extract. After full development of plates in solvent systems, plates were dried at room temperature. Then, one set of plates were immersed in iodine vapours in a glass jar. Second set of plates were sprayed with Vanillin-sulphuric acid spray. Both media used facilitated the detection of bioactive constituents in applied extract. This was carried out according to the method of Stahl, (1969).

Solvent System Applied.

The following solvent systems were tested to develop the TLC plates according to the method of Stahl, (1969). Chloroform / hexane / acetic acid (50:50:1) Chloroform / ethyl acetate / acetic acid (50:50:1) Methanol and chloroform (20: 80)

Test Organism

T. evansi was obtained from the Division of Parasitology, Indian Veterinary Research Institute, Izatnagar and was maintained in the laboratory by serial sub-passage in Swiss albino mice. The strain was routinely tested for virulence following the method of Williamson *et al.* (1982).

In Vitro Tryponocidal Activity

In vitro trypanocidal activity was carried out by modified method of Oliveira *et al.* (2004). In this method, a Vero cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) in 96-well flat bottom micro culture plates (Nunc, Denmark). Each well received 100 µl of DMEM containing 5x105 cells/ml. The plates were incubated at 37 C under 5% CO_2 for 48h to complete development of monolayer. After the formation of confluent monolayer, the medium (DMEM) was discarded and replaced with a fresh DMEM. And the medium was supplemented with 20-40% fetal calf serum (FCS), Gibco USA and antibiotics (100 units penicillin, 100 µg streptomycin and 40 µg gentamycin). A high parasitaemic blood from mouse was diluted with DMEM to obtain a final parasite of $1x10^6$ parasites/ml. The suspension (100 ml of medium with parasites) was added at rate of 1:1 to test MPE and the plate was incubated under same conditions mentioned above. The test was repeated at least thrice.

Stock of test MPE was solubilized in 1% dimethylsuphoxide (DMSO). The concentration in the experiment had no deleterious effect by itself on host cells or parasites.1% DMSO in distilled water was used as control (Young, 2000)

Infectivity Assessment

After incubation for antitrypanosomal activity was completed, contents of ELISA wells with reduced and apparently killed parasites from MPE of *K. senegalensis* root bark (0.1ml/mouse) was inoculated into six mice/group intraperitoneally and observed for more than 60 days for parasitemia (Petama, 1964).

In vitro Cytoxicity Test

Cytotoxic effect of the plant extract was determined according to the method described by Sidwell and Hoffman (1997). Vero cell line was grown as stated previously but was not supplemented with FCS because trypanosomes that required it were not involved. Confluent monolayer of Vero cell was treated with serial dilutions of test MPE (1.56-100 µg/ml) in triplicate and incubated under same conditions described previously. After 24 h of incubation, the culture plate was observed for evidence of cytotoxic effects such as distortion, detachment, swelling and sloughing of cells. The plate was incubated for 72 h and observed daily. It was repeated thrice.

In each case, after the 72 h of incubation, the culture media of the incubated Vero cells were discarded. The adhered cells were stained with a drop of crystal violet in phosphate buffered solution. The plate was incubated for 24 hours at 37°C in an ordinary incubator. The plate was observed for cytotoxic effects under inverted microscope.

Statistical Analysis

Results of trypanocidal activity were expressed as mean ± SEM. Statistical significance was determined by Sigma Stat (Jandel), USA.

RESULTS

The result of trypanocidal activity of MPE of *K. senegalensis* root bark was as given in table 1. MPE of *K. senegalensis* root bark exhibited immobilization and killing of trypanosomes in corresponding wells. At 250 µg/ml of *K. senegalensis* extract, there was drastic reduction of average means trypanosomes counts from initial concentration (40±0.0) to (2.667±0.88) at 5th h and completly killed of trypanosomes at 6th h of incubation in corresponding wells At concentrations of 500-1000 µg/ml of MPE of *K .senegalensis*, there was a progressive reduction and killing of trypanosomes in response to increased in concentrations of extract. *In vitro* cytotoxicity effects such as distortion, detachment, swelling, sloughing and death of cells from bottom of affected wells were observed as shown in Table 2. An average mean parasites count of 37.67± 0.58 was statistically critical value. Average mean parasites counts from 37.67± 0.58 and below was significant between the tested extracts and negative control with significant difference ($P \leq 0.05$). Infectivity assessment showed that mice inoculated with contents of ELISA wells where all trypanosomes appeared killed survived for more than 60 days. But other group of mice inoculated with contents of ELISA wells with reduced trypanosomes died of parasitaemia. In *in vitro* cytotoxicity test, swelling, distortion, detachment, sloughing and death of cells were observed in both MPE of *K. senegalensis* and DA

DISCUSSION

Methanol used in extraction of *K. senegalensis* root bark appeared suitable. This was demonstrated by the presence of bioactive constituents as observed on TLC plates (plates not shown). Perhaps, other solvents will be suitable for its extraction as well Solvent system methanol/chloroform (20:80) was more suitable in development of TLC plates than other solvent systems tested as mentioned above. These results are comparable to extraction and development of MPE of *Picrorrhiza kurroa* rhizomes on TLC plates (Shaba *et al* 2007).

At 250 µg/ml, trypanocidal activity of MPE of *K. senegalensis* tree bark at 6 h of incubation is equivalent to 4 h of diminazine aceturate (standard drug). This indicates the potency of MPE of *K. senegalensis* in comparison to pure compound of diminazine aceturate. This result is comparable to *in vitro* trypanocidal activity of MPE of medicinal plants used in treatment of trypanosomosis in northern Nigeria with effective concentration at 8 mg/ml (Wurocheke and Nok, 2004), and MPE of *Picrorrhiza korroa* rhizomes where trypanosmes were completely killed at 500 µg/ml. (Shaba *et al.* 2007).

Both MPE of *K. senegalensis* and DA were cytotoxic to Vero cells at all concentrations except 3.13-1.56 and 6.25-1.56 µg/ml. Diminazine aceturate (standard reference drug) was only one concentration better than MPE of *K. senegalensis* as observed in *in vitro* cytotoxicty test. The cytotoxic effects of MPE of *K. senegalensis* on Vero cells are comparable to cytotoxic effects of MPE *of Terminalia arjuna* bark on human hepatoma cell line (HEPG2) with distortion and apoptosis of cells (Sarveswaran *et al.*, 2006) and MPE of *Plumbago zeylanica*, root bark with similar cytotoxic effects as *K. senegalensis* (Shaba *et al* 2006), respectively.

This is initial screening of *K. senegalensis* root bark against *T. evansi.* Therefore, it is difficult to determine exact bioactive constituents responsible for its antitrypanosomal activity and mechanism of action at this stage of preliminary screening. However, antitrypanosomal activity of MPE of *K. senegalensis* could be due to any of already isolated bioactive principles mentioned above. Mechanism of

action may be due to intercalation of MPE of *K. senegalensis* with DNA leading to death of trypanosomes, blockage of glycolysis pathway and interference with flagella which temporarily immobilizes trypanosomes (Denise and Barret, 2001).

Table 1. *In vitro* trypanocidal activity of methanolic extract of *Khaya senegalensis* tree bark against *Trypanosoma evansi on* Vero cell line

Concentration of plant extract in µg/ml	1 h	2 h	3 h	4 h	5h	6 h	7 h	8 h	9 h
250	31.00± 0.58	24.33± 0.33	15.67± 0.67	10.33± 0.33	2.667± 0.88	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
500	26.67± 0.67	18.67± 0.33	10.67± 0.33	2.667± 0.33	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
750	22.33± 0.33	13.67± 0.33	8.333± 0.67	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
1000	19.67± 0.33	8.667± 0.33	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0±0.0	0.0± 0.0	0.0± 0.0
Berenil (50)	22.00± 0.0	9.333± 0.33	1.333± 0.33	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0±0.0	0.0± 0.0	0.0± 0.0
Control (Negative control)	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00± 0.0	40.00±0 .0

Table 2. *In vitro* cytotoxic effect of methanolic extract of *Khaya senegalensis t*ree bark on Vero cell line compared to diminazine aceturate (Berenil)

Concentration of test material in µg/ml	Cytotoxic effects of extract and DA at various time intervals of incubation						
	24 h		48 h		72 h		Control
	K. senegalensis	DA	*K. senegalensis*	DA	*K. senegalensis*	DA	
100	100%	66.6%	100%	100%	100%	100%	0
50	100%	33.3%	100%	100%	100%	100%	0
25	66.6%	0	100%	33.3	100%	66.6	0
12.5	33.3%	0	66.6%	0	66.6	33.3%	0
6.25	0	0	0	0	33.3%	0	0
3.13	0	0	0	0	0	0	0
1.56	0	0	0	0	0	0	0

Same concentrations were used for both MPE of K. senegalensis and DA. Cytotoxicity effects increased with period of incubation depending on the concentrations of the extract and DA. ***Abbrev: DMSO*** *- Dimethysulfoxide;* ***MPE*** *- Methanolic plant extract;* ***DMEM*** *- Dulbecco's Modified Eagle Medium;* ***DA*** *- Diminazine aceturate*

The lowest concentration of MPE of *K. senegalensis* used in this experiment was 2I50 µg/ml in which there was immobilization and killing of trypanosomes. Lower concentrations may give the same results at later hours of incubation. Purification of the extract will remove cytotoxic portion of MPE of *K. senegalensis.* Purified potent portion of K. *senegalens* is responsible for trypanocidal action will show activity at lower concentrations and lower values of cytotoxicity test will be observed. Moreso, direct contact of mammalian cells with plant extracts gives an insight of its effects when it will be administered

in vivo. Physiologically, impacts of toxic portion of plants extracts administered *in vivo* differ from that of *in vitro* cytotoxicity test that mimic the same activity. *In vivo* testing of plants extracts are less severe compare to that of *in vitro* testing on Vero cells, which are pour directly on the cells, e.g. Diminazine aceturate (standard drug). Diminazine aceturate is cytotoxic to Vero cells as observed. But, Diminazine aceturate does not cause serious damage to body cells if used at appropriate concentrations. Despite Diminazine aceturate cytotoxic effects, which are less compare to its beneficial activities against susceptible parasites, it is largely used in treatment of trypanosomosis.

In conclusion, results of current investigation show a potential trypanocidal compound(s) from *K. senegalensis* tree bark. Further research is required (e.g. bioassay-guided purification/ *in vivo* test.) to isolate the compound (s) responsible for its trypanocidal activity.

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